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BRIEF COMMUNICATION

Microsatellite markers designed for tree-fern species *Dicksonia sellowiana*

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Abstract

Microsatellite markers were developed for *Dicksonia sellowiana* (*Dicksoniaceae*), an overexploited and endangered tree-fern species native to Brazil. From an enriched genomic library, 11 primer pairs were selected and used to characterize 36 *D. sellowiana* individuals from six Brazilian populations. Eight primer pairs amplified dinucleotide and hexanucleotide repeats with two to ten alleles per locus; three primer pairs were monomorphic. For the set of polymorphic markers, the mean observed and expected heterozygosity ranged from 0.29 to 0.44 and from 0.27 to 0.56, respectively. Eight of the primer pairs were also successfully amplified for *Cyathea vestita* (*Cyatheaceae*). These molecular markers can be useful tools for genetic studies aiming to analyze the impact of deforestation and overexploitation on the population structure and genetic diversity of fern species from various botanical families.

Additional key words: *Cyathea vestita*, molecular markers, *Pteridophyta*, simple sequence repeat, xaxim.

There are an estimated 1 196 native Brazilian fern species belonging to 122 genera (Prado and Sylvestre 2011), most of which are poorly understood. The best known tree-fern species is xaxim (*Dicksonia sellowiana* Hooker, *Dicksoniaceae*, *Pteridophyta*) which has a natural distribution throughout Central and South America and can be found in seasonally wet habitats. In Brazil, *D. sellowiana* occurs in remnants of the dense Atlantic rainforest and *Araucaria* forests (Condack 2011). The Atlantic rainforest biome has been dramatically reduced to 22 % of its original area and the remaining fragments are in various stages of regeneration. Only 7 % of the remaining Atlantic rainforest is relatively well preserved in fragments larger than 10 000 m^2.

Since 1992, *D. sellowiana* has been on the list of Brazilian endangered species as a result of over-exploitation due to the popularity of xaxim in the craft and industry. Since 1977, *Dicksoniaceae* have been included in Appendix II of CITES (Convention on International Trade in Endangered Species). Despite the fact that *D. sellowiana* is relatively well studied, researchers have not yet determined whether reproduction occurs through intragametophytic, intergametophytic, or intersporophytic breeding, or a combination of these systems due to a lack of molecular or other systematically collected data. Studies in ecological genetics are urgently required to inform conservation policies for remnant populations of this fern and other threatened

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Abbreviations: CITES - Convention on International Trade in Endangered Species; CTAB - cetyltrimethylammonium bromide; PCR - polymerase chain reaction; SSR - simple sequence repeat; TBE - Tris/borate/EDTA.
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plant species.

Simple sequence repeats (SSR) or microsatellite markers have been developed and used as an effective tool in genetic approaches (Hou *et al*. 2011, Guidugli *et al*. 2012, Santos *et al*. 2012). Even when classified as monomorphic on the base of their length, as long as they present sequence variation, microsatellites can be used for a diverse range of analyses (Nazareno and Reis 2011). Despite their utility, microsatellite markers have not yet been developed for any fern species native to Brazil. In order to investigate genetic and ecological processes in *D. sellowiana* populations, 11 microsatellite markers were isolated and validated for this species. We further investigated the transferability of the loci to *Cyathea vestita* Mart., another tropical tree-fern species from the *Cyatheaceae* family.

A microsatellite-enriched library was constructed according to the method employed by Billotte *et al.* (1999) using biotin-labeled oligoprobes and streptavidin-coated magnetic beads. Genomic DNA was extracted from the leaf tissue of one individual of *D. sellowiana* following the standard cetyltrimethylammonium bromide (CTAB) procedure (Alzate-Marin *et al*. 2009). Genomic DNA (500 ng) was digested with *RsaI*. The fragments were then linked to adapters (*Rsa21* 5'-CTC TTGCTTACGCGTGGACTA-3' and *Rsa25* 5'-TAGTCC ACGCGTAAGCAAGAGCACA-3'). These fragments were enriched for dinucleotide sequences using the biotinylated probes $(CT)_8$, $(GT)_8$, and $(TTC)_8$. Streptavidin-coated paramagnetic beads (streptavidin *Magne-Sphere* paramagnetic particles, *Promega*, Madison, WI, USA) were used to recover complementary Rsa-fragments. Recovered fragments were amplified by polymerase chain reaction (PCR), using the *Rsa21* adapter as a primer, and linked to the *pGEM-T Easy Vector* sequence (*Promega*). Plasmids were introduced into *Escherichia coli* XL-1 Blue strain (*OmniMAX 2T-1*, *Invitrogen*, Carlsbad, CA, USA) and the cells were recovered and grown on selective Luria-Bertani agar containing ampicilin (100 µg dm^{-3}) and X-galactosidase (5-bromo-4-chloro-indolyl-ß-D-galactoside; 50 µg dm^{-3}). A total of 96 clones were sequenced using the *DYEnamic ET Dye Terminator* kit (*GE Healthcare*, Buckinghamshire, UK) in a *MegaBACE* sequencer (*GE Healthcare*). The online software for microsatellite marker development (*WebSat*) (Martins *et al.* 2009) was used to identify di-, tri-, tetra-, and hexa-nucleotides with seven or more repeats and the primers were designed with the software *Primer3Plus* (Untergasser *et al*. 2007).

Every reaction consisted of a final volume of 0.015 cm^3 containing 0.5 µM of each primer, 0.3 unit of *Taq* DNA polymerase (*Invitrogen*, Carlsbad, CA, USA), 0.25 mM of each dNTP, 1.0 mM $MgCl_2$, 15 ng of template DNA, and 1× PCR buffer [75 mM Tris-HCl, pH 9.0, 50 mM KCl, and 20 mM $(NH_4)_2SO_4$]. The PCR profile used to amplify the microsatellites was 94 °C for 3 min; 30 cycles of denaturation at 94 °C for 30 s, annealing at different temperatures (Table 1) for 45 s, 72 °C for 1 min, and a final elongation step at 72 °C for 10 min, performed in a *Veriti® 96-Well* Thermal Cycler (*Applied Biosystems*, California, USA). PCR products were denatured and separated in 10 % (m/v) denaturing polyacrylamide gels stained with silver nitrate. Gels were run with 1× TBE buffer on a vertical electrophoresis apparatus at a constant electric current (21 mA for each gel). Product sizes were determined by comparison with a 10 base pair DNA ladder (*Invitrogen*).

To evaluate polymorphism, 36 individuals of *D. sellowiana* were sampled from six populations (six individuals per population) located in the south and southeast regions of Brazil (Espírito Santo, Rio de Janeiro, São Paulo, Paraná, Santa Catarina, and Rio Grande do Sul). Distances between populations ranged from 190 to 1 400 km. In order to reduce the risk of illegal harvesting, the exact locations of these populations are not included. Genetic diversity parameters and probabilities of paternity exclusion were estimated using *CERVUS version 3.0* (Kalinowski *et al*. 2007). Tests for the genotype independence across loci were performed using *FSTAT* software (Goudet 2002) for all pairs of loci within and among populations applying the Bonferroni correction for multiple comparisons. Given the sample size, the Hardy-Weinberg equilibrium calculation was not performed.

Of the 20 primer pairs, 11 amplified fragments of the expected sizes (Table 1) whereas the other nine primer pairs showed no amplification, multibanding patterns or pronounced stutters. Among the 11 selected, three loci were monomorphic with fixed alleles in all sampled *D. sellowiana* populations. These monomorphic loci may be useful for other populations or related taxa, or screened for sequence variations in future trials. For the eight polymorphic loci, a total of 38 alleles were identified and the mean number of alleles per population ranged from 1.8 to 3.1 (Table 2). Mean observed and expected heterozygosity in population ranged from 0.29 to 0.44 and from 0.27 to 0.56, respectively (Table 2). For the set of populations, the number of alleles per locus ranged from two (*Dic*10) to 10 (*Dic*01), and the observed and expected heterozygosities ranged from 0.08 to 0.78 and from 0.34 to 0.84, respectively. The paternity exclusion probability, corresponding to the power with which a locus excludes an individual of being the parent of an offspring, reached 0.86 for the first parent and 0.98 for the second parent using the polymorphic loci. No significant linkage disequilibrium ($P > 0.001$) was found within or among populations. Eight of the 11 primer pairs developed for *D. sellowiana* were successfully transferred to *Cyathea vestita* (Table 1) using the same PCR protocol described above. This result indicates a significant potential for transferring these microsatellite markers to other fern species of different botanical families.

The microsatellite markers described herein are useful for genetic investigations of *D. sellowiana*. These markers will be used to evaluate the genetic diversity of Brazilian populations of this endangered species. Similar analyses can also be conducted using the same set of markers for related taxa.

Table 1. Characteristics of 11 microsatellite loci developed for *Dicksonia sellowiana* from the south and southeast Brazil including: loci names, GenBank accession numbers, primer sequence (F: forward, R: reverse), repeat motif, fragment size, annealing temperature (Ta), and the success of transferability (T) to *Cyathea vestita* indicated by (+).

Dicksonia sellowiana						*Cyathea vestita*	
Loci	acc. Nos.	sequence 5' - 3'	repeat motif	Size [bp]	Ta [ºC]	T	Ta [ºC]
Dic01	JX423820	F: GCGAGAGAGAGCAAGAGGAA R: TGGCCTTTCACACAGAAGC	$(TG)_{20}...(TG)_8$	278	58	+	56
Dic02	JX423821	F: TGCAATGATGTTGGTGTTTATG R: TCCTCTCCCTCGTGCTACTC	$(TC)_7(AC)_{11}$	211	52	+	53
Dic03	JX423822	F: CCTCCCACTTAAAGGCAATG R: TACGGGGGATGGTGATGTA	$(TACACA)_9$	241	55		
Dic04	JX423823	F:CACATAGAATAAACATGCCTTTGC R: CTCAGGGTCTCACCACCTTC	$(CA)_{17}$	145	50	+	56
Dic06	JX423824	F: TTGCTTCCCTTTGTCATGC R: GCGACTAAGCGTTTTTAGGC	$(CT)_{13}(CA)_{13}$	288	54	+	50
Dic08	JX423825	F:TCCCTCACATACACGCACAC R: CACCCCTTCATCTCACATCC	$(CA)_{11}$	219	58	+	53
Dic10	JX423826	F:GGAGATAGTGAGTAAGAGAGAGAAAGC R: GTCTTGTTGGAAATAAAGGGGA	$(AG)_{17}$	272	60	+	55
Dic11	JX423827	F: CCACCTGCTACCTCCAATGT R: GCAAATTCAGCCATCCATCT	$(CA)_{10}$	164	58		
Dic12	JX423828	F: CCATTGGGTCTTTTGCAGAC R: CCATGCAACGGTTTAAGAGC	$(CT)_{15}$	272	56		
Dic13	JX423829	F: AGAAGAAAATTCACCCACCATC R: AGAATGAGGGGGAGAGGAAG	$(TG)_9$	111	55	+	50
Dic14	JX423830	F: GGAGAGGGAGAGCTTCAACT R: AAGAAAACTCAGTCATCCGTCT	$(CA)_9$	105	50	+	58

Table 2. Size range [bp], number of samples (n) per population, number of alleles (A), and observed (H_O) and expected (H_E) heterozygosity for eight polymorphic microsatellite loci from *Dicksonia sellowiana* populations from the south and southeast of Brazil (ES - Espírito Santo, RJ - Rio de Janeiro, SP - São Paulo, PR - Paraná, SC - Santa Catarina, and RS - Rio Grande do Sul).

Loci	Size range	ES (n = 6)			RJ (n = 6)			SP (n = 6)			PR (n = 6)			SC (n = 6)			RS (n = 6)		
		A	H_O	H_E	A	H_O	H_E	A	H_O	H_E	A	H_O	H_E	A	H_O	H_E	A	H_O	H_E
Dic01	262-286	2	0.00	0.53	6	0.50	0.88	5	0.33	0.83	4	0.67	0.75	4	0.50	0.83	6	0.67	0.90
Dic02	207-215	2	0.17	0.17	3	0.17	0.33	3	0.50	0.70	2	0.00	0.60	3	0.00	0.60	2	0.00	0.33
Dic03	235-259	2	1.00	0.50	3	0.33	0.57	3	0.33	0.55	4	1.00	0.77	4	0.67	0.63	4	0.50	0.77
Dic06	288-292	2	0.83	0.50	2	0.67	0.47	2	1.00	0.50	2	1.00	0.50	3	0.67	0.73	3	0.50	0.63
Dic08	215-223	2	0.33	0.30	3	0.17	0.57	2	0.00	0.33	2	0.00	0.33	1	0.00	0.00	2	0.67	0.47
Dic10	272-274	1	0.00	0.00	2	0.00	0.33	2	0.00	0.33	2	0.33	0.30	2	0.17	0.43	2	0.00	0.60
Dic11	162-172	1	0.00	0.00	2	0.00	0.33	1	0.00	0.00	3	0.50	0.57	3	0.67	0.68	3	1.00	0.58
Dic12	268-276	2	0.17	0.17	4	0.83	0.73	2	0.17	0.17	2	0.17	0.17	2	0.33	0.30	2	0.17	0.17

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